

Molecular cloning and expression of a novel truncated form of chicken *trkC*

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The *trk* family of tyrosine protein kinase genes serves crucial roles for the development of the nervous system and the survival of neurons. The members of this gene family, *trk*, *trkB* and *trkC*, bind a distinct neurotrophin of the nerve growth factor (NGF) gene family, and trigger the intracellular signals which elicit trophic and differentiating effects on neurons. Adding to these neurotrophic receptor kinases, the truncated forms without the tyrosine kinase domain have been cloned and characterized. It has been thought that the existence of truncated forms is limited to *trkB*; however, very recently the truncated *trkC* has been cloned in rat [(1993) Neuron 10, 963–974; (1993) Neuron 10, 975–990]. We independently approached and molecularly cloned a truncated form which belongs to the chicken *trkC*. The truncated *trkC* possesses the binding and the transmembrane domains but not the tyrosine kinase domain. Northern blot analysis shows that the truncated form is preferentially expressed in the adult central nervous system. The truncated form is scarcely expressed during the embryonic stages. The conservation of the truncated *trkC* beyond species suggests they have specific functions.

trkC; cDNA cloning; Tyrosine kinase; Expression

1. INTRODUCTION

Neurons can be distinguished from the other cells due to their extremely long survival without cell division. This specific characteristic is essential for the stable function of the neural network. The trophic effect by humoral factors would play an important role for the survival of neurons. Among the trophic factors, neurotrophins, which belong to the nerve growth factor (NGF) gene family, NGF [1], brain-derived neurotrophic factor (BDNF) [2,3], neurotrophin-3 (NT-3) [4–9], NT-4 [10], and NT-5 [11], have been analyzed most extensively. These neurotrophins enhance the survival of cultured neurons and promote their differentiation [12]. In vivo application of NGF to embryos partially prevents the programmed neuronal death in the dorsal root ganglia [13], while the administration of anti-NGF antibody accelerates their death [14,15]. The signal transduction triggered by neurotrophins, has been uncovered rapidly in recent years. Identification of the *trk* gene family as the receptors for neurotrophins clearly demonstrates that the tyrosine phosphorylation initiates the intracellular neurotrophic signals [16–18], although it is not known whether the *trk* molecule alone can compose the high affinity receptor and transfer the necessary information ([19,20] vs. [21]).

So far, four members of the *trk* family; *trk* [16], *trkB* [22], *trkC* [23], and *Dtrk* [24] have been characterized in mammals and *Drosophila*. Each member of the *trk* receptor family has a distinct binding spectrum to neurotrophins and a different expression pattern in the nervous system [25], except that *Drosophila trk* (*Dtrk*) may function as an adhesion molecule. In addition to these relatives of *trk*, truncated forms which lack the tyrosine kinase domain are transcribed from the mouse [26] and rat *trkB* genes [27]. In spite of this finding in *trkB*, *trkC* has been considered to encode a single type of transcript from the Northern blotting observation [23]. However, very recently, the truncated molecule was reported in the mammalian *trkC* [28,29]. In this study, we independently isolated the truncated form of chicken *trkC* which lacked the tyrosine kinase domain. Our independent approach confirmed the existence of truncated *trkC* in chicken, suggesting unknown functional importance of the truncated form of *trkC* beyond species.

2. MATERIALS AND METHODS

2.1. Isolation and characterization of chicken *trkC* cDNA clones

For the screening of the cDNA library, we amplified DNA fragment of the porcine *trkC* [23] using the reverse transcriptase PCR (RT-PCR) technique as described previously [30]. The amplified fragment was subcloned into the pBluescript (Stratagene, USA). The sequence was checked with the automatic sequencer (Applied Biosystem, USA), and then radio-labeled with a random primer labeling kit (Boehringer-Mannheim, Germany) and [³²P]dCTP (NEN Research Products, Dupont). Hybridization was done in 6 × SSC, 5 × Denhart's solution, 1% SDS, and 100 μg/ml of denatured salmon sperm, at 50°C. Filters were washed in 6 × SSC, 0.1% SDS at 50°C 3 times over. 5 × 10⁵ to 1 × 10⁶

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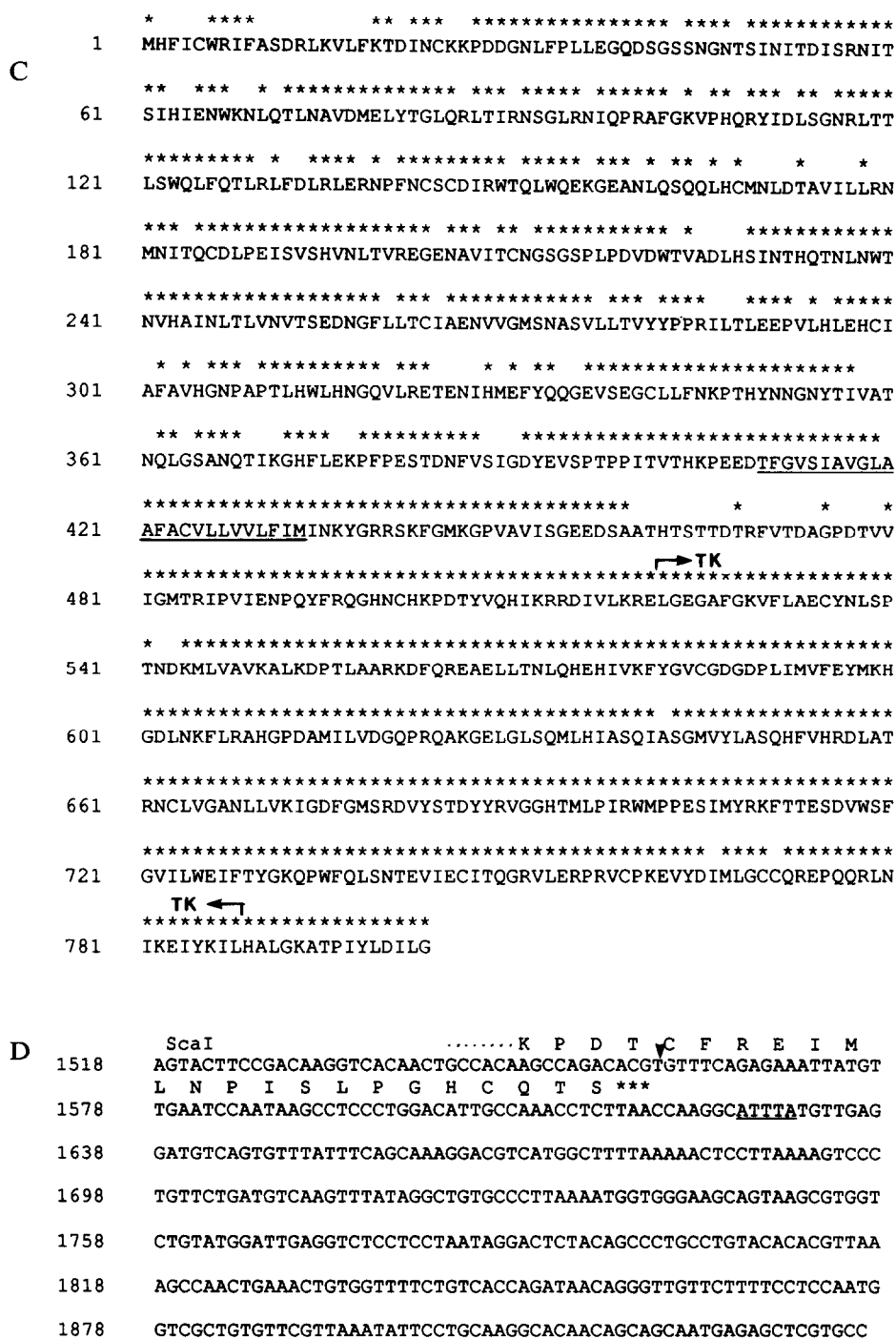


Fig. 1. (A) Schematic diagram showing the structures of the *trkC*-1, -2 and -3 cDNA clones. The boxes represent the coding regions of the cDNAs. The restriction sites of *Bgl*II (G), *Nhe*I (N), *Pst*I (P), *Sca*I (S), *Xho*I (X) are indicated. The *trkC*-3-specific region is indicated with a dotted box and a thick bar. The transmembrane domains (TM) are indicated with black boxes. The tyrosine kinase domains (TK) are indicated with shaded boxes. (B) The composite cDNA of *trkC*-1 and -2 includes an 803 amino acid open reading frame. The transmembrane domain is surrounded with a box. The tyrosine kinase domain spans between arrows. The stop codon at the end of the open reading frames are indicated with (***). Classical polyadenylation signals are not found within the non-coding sequence. AUUUA box [39] is underlined. The arrowhead indicates the truncating site to the *trkC*-3 type of transcript. These nucleotide sequences were analyzed with automatic sequencer (Applied Biosystems, USA) using the dye primer and dye terminator *Taq* sequencing kits delivered from the same company. (C) The identity of amino acid sequences between chicken *trkC* and porcine *trkC*. The identical amino acids are indicated with stars (*) above the amino acid sequence of chicken *trkC*. The tyrosine kinase domain is indicated with arrows and TKs. The transmembrane domain is underlined. (D) The *trkC*-3 specific nucleotide and amino acid sequences are shown. The truncating site is indicated with an arrow head. The AUUUA box is underlined.

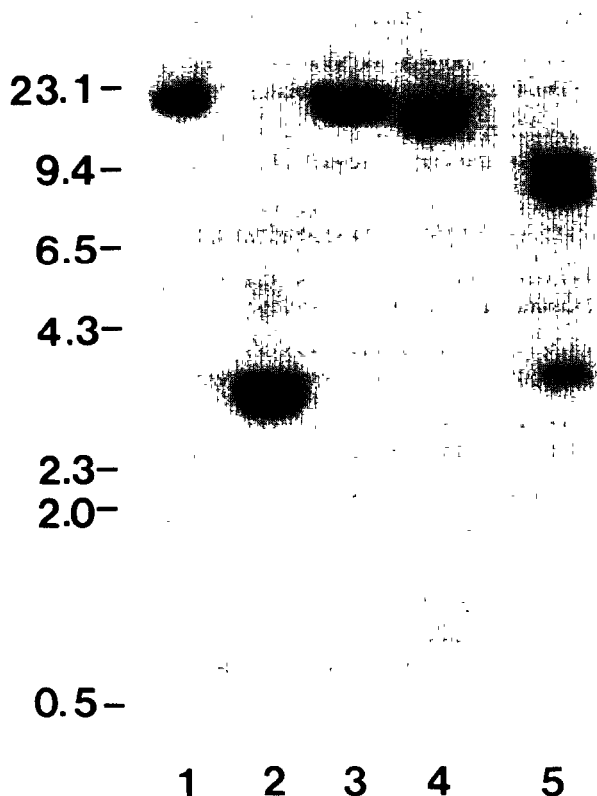


Fig. 2. Southern blottings of chicken genomic DNA probed with chicken *trkC*-1 cDNA (lanes 1–4) and with rat *trkB* cDNA (lane 5). DNAs were digested with *Xba*I (lanes 1 and 5), *Hind*III (lanes 2), *Eco*RI (lanes 3) and *Bam*HI (lanes 4). 10 μ g of digested DNAs were separated on 0.7% agarose gel, blotted to a nylon membrane (Hybond-N, Amersham, USA). The filter was hybridized with *trkC*-1 cDNA (lane 1–4) and with rat *trkB* cDNA (lane 5). The hybridization and washing were performed in a highly stringent condition as described previously [33]. Briefly, the filter was hybridized in $5 \times$ SSC, 50% formamide, 1% SDS at 42°C and washed finally in $0.1 \times$ SSC, 0.1% SDS at 60°C for 30 min. In the case of rat *trkB* probe, the same filter was hybridized in $5 \times$ SSC, 40% formamide, 1% SDS at 38°C. Final washing was performed in $1 \times$ SSC at 50°C. Full-length cDNA of rat *trkB* was amplified with two sets of RT-PCR reactions which amplified divided portions of rat *trkB* cDNA as described previously [30]. The PCR products were subcloned into pBluescript and used for the hybridization after confirming the sequence.

not extensively conserved. The two cysteines near the amino-terminal were lacking in the chicken *trkC*, leaving the necessity to test the binding characteristic of this molecule to neurotrophins.

Genomic Southern blot analysis probed with the *trkC*-1 cDNA showed only a single band of strong intensity in each digestion by restriction enzymes (Fig. 2). Supportingly, the same pattern was observed with the porcine *trkC* probe amplified by RT-PCR (data not shown). The bands detected with the rat *trkB* probe were definitely different (Fig. 2). These findings indicate that our cDNA clones actually encode the chicken *trkC*.

We conducted Northern blot analysis with the composite cDNA of *trkC*-1 and *trkC*-2 as the probe. Sur-

prisingly, the blot showed multiple bands from 9.0 kb to 0.4 kb in the stringent condition (Fig. 3A, lane 5). The bands which hybridized with the chicken *trkC* probe were clearly different in their sizes from the *trkB* bands (Fig. 3A, lanes 5 and 6). This denied the possibility of cross-hybridization. The bands smaller than 1.2 kb are incapable of encoding the ordinary size *trkC* protein. Thus we suspected that there might be some truncated forms of *trkC*. We re-screened the cDNA library with the *trkC*-1 cDNA probe and obtained four independent clones which encode an identically truncated *trkC*. The sequence of a representative cDNA (*trkC*-3) is shown (Fig. 1D). The truncated *trkC* lacked the tyrosine kinase domain. The sequence around the truncation site (indicated with an arrowhead in Fig. 1D) was compatible with the splicing consensus. The same character is observed in the rat and mouse *trkB*s and *trkC*s at their truncation sites [26–29]. Read-through of the splicing site will be the common mechanism to produce these truncated forms [35]. With the direct sequencing of the PCR product, we confirmed that the genomic sequence around the truncation matched exactly to that of cDNA (data not shown). These data clearly indicated that the *trkC*-3 type cDNA is not a cloning artifact but an alternatively spliced transcript which encoded the truncated *trkC* without the tyrosine kinase domain.

Northern blot analysis with the different cDNA probes was performed to define the gross structures of various transcripts (Fig. 3A). Different to the first speculation, the *trkC*-3 type of truncated form was derived from the 4.0 kb transcript, because it hybridized with the amino-terminal probe (Fig. 3A; lane 3) as well as the *trkC*-3 specific probe (Fig. 3A; lane 4), but not with the *trkC*-1 specific probe (Fig. 3A; lane 2). Since the *trkC*-3 transcript does not encode the tyrosine kinase domain, its product will not transfer the signals at least by the direct tyrosine phosphorylation of target proteins. The homology search with Mac Vector II could not find any consensus for the other signal transductions in the intracellular region of the *trkC*-3.

The bands of 7.2 kb and 4.8 kb hybridized with both with the carboxyl-terminal probe of *trkC*-1 (Fig. 3A; lane 2) and the amino-terminal probe (Fig. 3A; lane 3). Thus these transcripts seem to encode the complete *trkC* receptor for the binding and the signal transduction. The 1.2 kb band hybridized all the probe used for the analysis (Fig. 3A). However, the signal intensities were obviously different; the 1.2 kb band hybridized strongly with the carboxyl-terminal probe (Fig. 3A; lane 2), but only weakly with the amino-terminal probe (Fig. 3A; lane 3). The 1.2 kb band showed a faint signal in lane 4, presumably because the probe 4 contained the common sequence to *trkC*-1 between the *Sca*I site and the truncation site. We could deny the non-specific hybridization to 1.2 kb band, firstly because this band did not hybridize with the other probes such as *trkB* and β -actin, and secondly because we used the highly stringent

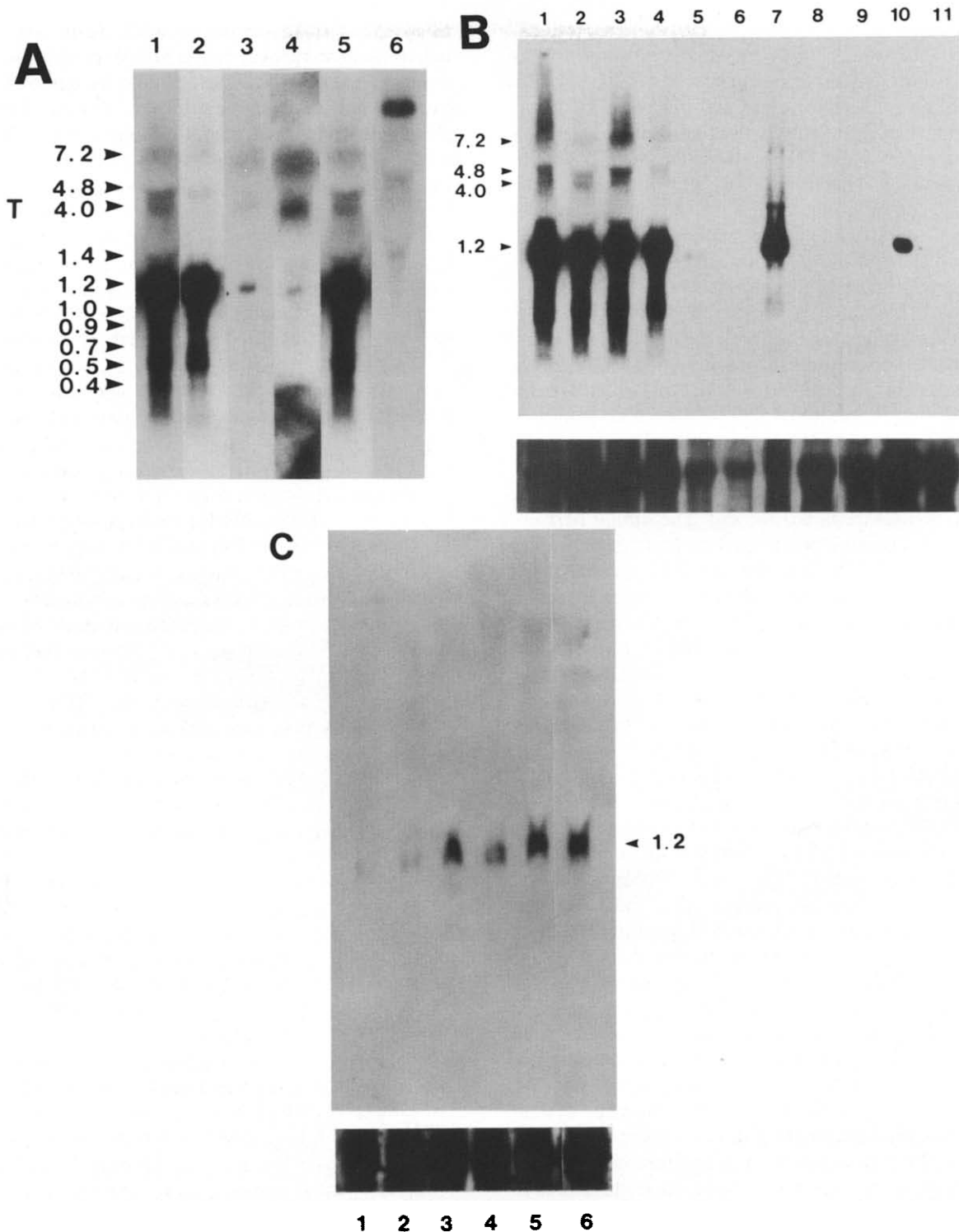


Fig. 3. (A) Structural analysis of the various *trkC* transcripts. A Northern blot filter of the adult whole brain mRNA was hybridized with the various cDNA probes of *trkC*. The lane number from 1 to 4 corresponds to the probe number in Fig. 1A. Lane 5 was hybridized with the composite cDNA of *trkC*-1 and *trkC*-2. Lane 6 was hybridized with chicken *trkB* cDNA (Okazawa et al., unpublished). The 4.0 kb truncated transcript is indicated with (T). About 10 μ g of RNA was loaded. RNAs were blotted to Hybond-N (Amersham, UK). (B) The expression of chicken *trkC* in various tissues. RNAs were prepared from cerebrum (lane 1), optic lobe (lane 2), cerebellum (lane 3), brain stem (lane 4), spinal cord (lane 5), roots of the lumbar plexus (lane 6), heart (lane 7), lung (lane 8), skeletal muscle (lane 9), spleen (lane 10), and ovary (lane 11) of an adult chicken. About 10 μ g of RNA was loaded on each lane. The filter was hybridized with the full-length cDNA composed from *trkC*-1 and *trkC*-2 clones. The 7.2 kb and 4.8 kb transcripts encoding the catalytic form and the 4.0 kb transcript encoding the truncated form are indicated (see text). The lower panel indicate the hybridization of the same filter with β -actin probe. We used the highly stringent condition for hybridization and washing as described previously [32]. (C) Developmental expression pattern of chicken *trkC* in the optic lobe is shown. mRNAs were prepared from the optic lobes of embryos of day 4 (lane 1), day 6 (lane 2), day 8 (lane 3), day 10 (lane 4), day 12 (lane 5) and day 16 (lane 6). About 10 μ g of RNA was loaded on each lane. The filter was hybridized with the composite full-length cDNA. The 1.2 kb transcript was up-regulated. The lower panel indicates the hybridization of the same filter with β -actin probe.

condition for the hybridization and the washing. We suspected that the 1.2 kb band might be composed of multiple but similar-sized transcripts. The bands smaller than 1.2 kb hybridized with the carboxyl-terminal probe (Fig. 3A; lane 2), but not with the amino-terminal probe (lane 3) nor with the *trkC*-3 specific probe (lane 4). Therefore, most of these short transcripts, including a part of the 1.2 kb complex, will be composed of the 3' non-coding region. Supportingly, we picked up short cDNA clones which possess only the 3' non-coding sequence (data not shown).

Northern blot analysis using the various tissues of adult chicken indicated that both the catalytic form and the truncated form were expressed specifically in the nervous system (Fig. 3B; lanes 1–3). Only exceptionally, did the brainstem basis not express the 4.0 kb truncated form (Fig. 3B; lane 4). We conducted preliminary *in situ* hybridizations using the antisense riboprobes. The specific probe for *trkC*-3 showed the signals around the lateral ventricles (data not shown). The similar periventricular hybridization is reported in the truncated form of mouse *trkB* [26]. The catalytic and the truncated forms were not expressed in the non-nervous tissues as well as in the peripheral nerve roots which contained mainly the Schwann cells (Fig. 3B). In some tissues (peripheral nerve root, lane 6; and spleen, lane 10), the 1.2 kb mixed transcripts were expressed preferentially. 5.8 kb and 2.0 kb bands were observed in the heart, although their structure has not been determined.

We analyzed the developmental expression pattern of chicken *trkC* in the optic lobe with Northern blotting (Fig. 3C). The clear up-regulation was observed in the mixed transcripts of 1.2 kb. However, the higher bands were scarcely detected. The longer exposure showed faint signals of these transcripts. This indicated that both the catalytic and the truncated form of *trkC* were expressed in very small amounts during the embryonic stages. Interestingly, at day 8, the expression of 1.2 kb band was decreased transiently. In the central nervous system, *trkB* is expressed more abundantly and from an earlier stage of the development than *trkC* (our unpublished observation). NT-3 [4–9], the ligand for *trkC* [23], is expressed adequately in the central nervous system throughout development and in the adult stage [36,37]. Thus the NT-3/*trkC* system may function mainly from a later stage of development to the adult stage. These data seem to suggest the importance of the NT-3/*trkC* system for the survival of mature neurons in physiological and possibly pathological conditions.

The real function of these truncated forms remains to be clarified. However, the existence of truncated *trkC* both in mammals and chicken seems to suggest that they serve some indispensable function. Their periventricular expression pattern seems to be compatible with the idea that the truncated form might function as a scavenger receptor or as a transporter [26]. The other possibility is that the truncated forms of *trks* might act

as competitors against the catalytic form, like a truncated epidermal growth factor (EGF) receptor [38]. This might be the case if a single neuron expresses both the catalytic and the truncated forms. We need further experiments to characterize the function of truncated *trkC*.

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REFERENCES

- [1] Levi-Montalcini, R. (1987) *Science* 237, 1154–1162.
- [2] Barde, Y.-A., Edgar, D. and Thoenen, H. (1982) *EMBO J.* 1, 549–553.
- [3] Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P., Thoenen, H. and Barde, Y.-A. (1989) *Nature* 341, 149–152.
- [4] Ernfors, P., Ibanez, C.F., Ebendal, T., Olson, L. and Persson, H. (1990) *Proc. Natl. Acad. Sci. USA* 87, 5454–5458.
- [5] Hohn, A., Leibrock, J., Bailey, K. and Barde, Y.-A. (1990) *Nature* 344, 339–341.
- [6] Johns, K.R. and Reichert, L.F. (1990) *Proc. Natl. Acad. Sci. USA* 87, 8060–8064.
- [7] Maisonnier, P.C., Belluscio, L., Squinto, S., Ip, N.Y., Furth, M.E., Linsay, R.M. and Yancopoulos, G.D. (1990) *Science* 247, 1446–1451.
- [8] Rosenthal, A., Goeddel, D.V., Nguyen, T., Lewis, M., Shih, A., Laramee, G.R., Nikolics, K. and Winslow, J.W. (1990) *Neuron* 4, 767–773.
- [9] Kaisho, Y., Yoshimura, K. and Nakahama, K. (1990) *FEBS Lett.* 266, 187–191.
- [10] Hallbook, F., Ibanez, C.F. and Persson, H. (1991) *Neuron* 6, 845–858.
- [11] Berkemeier, L.R., Winslow, J.W., Kaplan, D.R., Nikolics, K., Goeddel, D.V. and Rosenthal, A. (1991) *Neuron* 7, 857–866.
- [12] Barde, Y.-A. (1989) *Neuron* 2, 1525–1534.
- [13] Hamburger, V., Brunso-Bechtold, J.K. and Yip, J.W. (1981) *J. Neurosci.* 1, 60–71.
- [14] Johnson Jr., E.M., Gorin, P.M., Brandeis, L.D. and Pearson, J. (1980) *Science* 210, 916–918.
- [15] Goedert, M., Otten, U., Hunt, S.P., Bond, A., Chapman, D., Schulumpf, M. and Lichtensteiger, W. (1984) *Proc. Natl. Acad. Sci. USA* 81, 1580–1584.
- [16] Martin-Zanca, D., Oskam, R., Mitra, G., Copeland, T. and Barbacid, M. (1989) *Mol. Cell. Biol.* 9, 24–33.
- [17] Kaplan, D.R., Hempstead, B.L., Martin-Zanca, D., Chao, M.V. and Parada, L.F. (1991) *Science* 252, 554–558.
- [18] Klein, R., Jing, S.Q., Nanduri, V., O'Rourke, E. and Barbacid, M. (1991) *Cell* 65, 189–197.
- [19] Hempstead, B.L., Martin-Zanca, D., Kaplan, D.R., Parada, L.F. and Chao, M.V. (1991) *Nature* 350, 678–683.
- [20] Berg, M.M., Sternberg, D.W., Hempstead, B.L. and Chao, M.V. (1991) *Proc. Natl. Acad. Sci. USA* 88, 7106.
- [21] Jing, S., Tapley, P. and Barbacid, M. (1992) *Neuron* 9, 1067–1079.
- [22] Klein, R., Parada, L.F., Coulier, F. and Barbacid, M. (1989) *EMBO J.* 8, 3701–3709.
- [23] Lambelle, F., Klein, R. and Barbacid, M. (1991) *Cell* 66, 967–979.
- [24] Pulido, D., Campuzano, S., Koda, T., Modolell, J. and Barbacid, M. (1992) *EMBO J.* 11, 391–404.

- [25] Chao, M.V. (1992) *Neuron* 9, 583–593.
- [26] Klein, R., Conway, D., Parada, L.F. and Barbacid, M. (1990) *Cell* 61, 647–656.
- [27] Middlemas, D.S., Lindberg, R.A. and Hunter, T. (1991) *Mol. Cell. Biol.* 11, 143–153.
- [28] Valenzuela, D.M., Maisonpierre, P.C., Glass, D.J., Rojas, E., Nunez, L., Kong, Y., Gies, D.R., Stitt, T.N., Ip, N.Y. and Yancopoulos, G.D. (1993) *Neuron* 10, 963–974.
- [29] Tsoulfas, P., Soppet, D., Escandon, E., Tessarollo, L., Mendoza-Ramirez, J.-L., Rosenthal, A., Nikolics, K. and Parada, L.F. (1993) *Neuron* 10, 975–990.
- [30] Okazawa, H., Murata, M., Watanabe, M., Kamei, M. and Kanazawa, I. (1992) *FEBS Lett.* 313 (2), 138–142.
- [31] Chomzinski, P. and Sacchi, N. (1987) *Anal. Biochem.* 162, 156–159.
- [32] Okamoto, K., Okazawa, H., Okuda, A., Sakai, M., Muramatsu, M. and Hamada, H. (1990) *Cell* 60, 461–472.
- [33] Okazawa, H., Okamoto, K., Ishino, F., Ishino-Kaneko, T., Takeda, S., Toyoda, Y., Muramatsu, M. and Hamada, H. (1991) *EMBO J.* 10, 2997–3005.
- [34] Kozak, M. (1987) *Nucleic Acids Res.* 12, 857–873.
- [35] Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1989) *Molecular Biology of the Cell*, 2nd edn., Garland Publishing, New York/London.
- [36] Maisonpierre, P.C., Belluscio, L., Friedman, B., Alderson, R.F., Wiegand, S.J., Furth, M.E., Lindsay, R.M. and Yancopoulos, G.D. (1990) *Neuron* 5, 501–509.
- [37] Ernfors, P., Merlio, J.-P. and Persson, H. (1992) *Eur. J. Neurosci.* 4, 1140–1158.
- [38] Redemann, N., Holzmann, B., von-Ruden, T., Wagner, E.F., Schlessinger, J. and Ullrich, A. (1992) *Mol. Cell. Biol.* 12, 491–498.
- [39] Shaw, G. and Kamen, R. (1986) *Cell* 46, 659–667.